THE RAPID ACTIVATION IN VITRO OF THE CHLOROPLAST FRUCTOSE 1,6-BISPHOSPHATASE FOLLOWED USING A NEW ASSAY PROCEDURE

Luciana ROSA*
Botany School, University of Oxford, South Parks Road, Oxford OX1 3RA, England

Received 21 August 1981; revision received 17 September 1981

1. Introduction

Chloroplast Fru-P_2ase is reversibly activated on illumination [1] principally by means of a light-dependent reduction of Fru-P_2ase by thioredoxin [2]. In addition, light-driven increases in the stromal pH and in the stromal concentrations of Mg^{2+} and Fru-1,6-P_2 [3,4] are essential for the full activity of the reduced enzyme [4]. Fru-P_2ase can be activated in vitro by incubating the enzyme either with thioredoxin reduced by DTT [5] or with DTT alone [6,7]. In both cases the full activation of Fru-P_2ase in vitro has been observed in a time similar to that required in vivo only with the additional presence during the activation period either of Fru-1,6-P_2 and Ca^{2+} [8] or of Fru-1,6-P_2, Ca^{2+} and Mg^{2+} [9]. However, this requirement for Ca^{2+} is difficult to reconcile both with the virtual absence of Ca^{2+} from the illuminated chloroplast stroma [10] and with the strongly inhibitory effect of Ca^{2+} on Fru-P_2ase activity [8,11].

Here, I report that for the full DTT-dependent activation of Fru-P_2ase in vitro in a time similar to that required in the intact chloroplast there is a requirement during the activation period for Mg^{2+} and for the continuous presence of Fru-1,6-P_2 but there is no requirement for Ca^{2+}. I have also introduced a new method for assaying Fru-P_2ase activity, in which the Fru-1,6-P_2 is regenerated during the assay.

Abbreviations: DTT, dithiothreitol; Fru-P_2ase, fructose 1,6-bisphosphatase (EC 3.1.3.11); Fru-1,6-P_2, D-fructose 1,6-bisphosphate, Fru-6-P, D-fructose 6-phosphate; PEP, phosphoenolpyruvate; U, enzyme units

* Permanent address: Istituto Botanico dell'Universita, Via Foria 223, Napoli, Italy

2. Materials and methods

All enzymes and other biochemicals were purchased from Sigma Chemical Co. (Poole). Fru-P_2ase purified as in [6] modified as in [7] and in both untreated and thiol-treated (DTT) form was a generous gift from S. A. Charles.

The activity of chloroplast Fru-P_2ase was measured from the rate of Fru-6-P formation from Fru-1,6-P_2. This was achieved by two different coupled assay procedures.

2.1. *Assay procedure A*

The reaction mixture contained in a total volume of 1 ml: 100 mM Tris–HCl (pH 8.0), 30 μM ethylene glycol bis-(β-aminoethyl ether), 5 mM $MgCl_2$, 0.5 mM NADP, 0.1 mM Fru-1,6-P_2, 50 mM DTT, 5 U phosphoglucose isomerase, and 2 U glucose-6-phosphate dehydrogenase. The reaction was started by the addition of 0.04 μg Fru-P_2ase. The increased A_{340} was followed at 25°C.

2.2. *Assay procedure B*

The reaction mixture contained in a total volume of 1 ml: 100 mM Tris–HCl (pH 8.0), 30 μM ethylene glycol bis-(β-aminoethyl ether), 5 mM $MgCl_2$, 0.1 mM Fru-1,6-P_2, 0.2 mM ATP, 0.2 mM phosphoenolpyruvate, 0.2 mM NADH, 50 mM DTT, 10 U lactic dehydrogenase, 10 U pyruvic kinase, and 2 U phosphofructokinase. The coupling enzymes were added in the order given. After the addition of lactic dehydrogenase there was a small decrease in A_{340} which corresponded to the pyruvate present as a contaminant in the PEP solution, and after the addition of phosphofructokinase and pyruvic kinase there was another small decrease in A_{340}, which corresponded

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

to the ADP present as a contaminant in the ATP solution. The reaction was started by the addition of 0.04 μg Fru-P_2ase, and the decreased A_{340} was followed at 25°C. When the A_{340} fell below a value corresponding at 0.1 mM NADH, further additions of PEP and NADH were made so as to bring their concentrations back to ~0.2 mM each.

At the concentrations used DTT did not affect either coupled assay system.

3. Results

Fru-P_2ase activity is generally assayed by procedure A. Here, I describe a new assay procedure, B. The advantage of procedure B is that the Fru-1,6-P_2 concentration is kept constant during the assay, thus resembling more closely the situation in vivo.

Similar rates of Fru-P_2ase activity were observed when similar samples of Fru-P_2ase were assayed with the two assay procedures (table 1). As observed in [7], the activated form of the enzyme (thiol-treated) gives a high activity even at low Fru-1,6-P_2 concentrations, while the inactive form (untreated) gives a high activity only when high concentrations of Fru-1,6-P_2 and Mg^{2+} are present (table 1). PEP was added at 0.2 mM in assay procedure B because higher concentrations inhibited to a slight, but significant, extent the Fru-P_2ase activity observed at low Fru-1,6-P_2 concentrations (table 1). Results similar to those in table 1 for the purified enzyme were obtained with a crude chloroplast extract that had been passed through a Sephadex G-25 column.

Table 1
Comparison between two assay procedures for the purified chloroplast Fru-P_2ase (μmol . min^{-1} . mg $protein^{-1}$)

Form of Fru-P_2ase	Additions	Assay procedure	
		A	B
Untreated	–	0	0
	Fru-1,6-P_2 2.5 mM + $MgCl_2$ 5 mM	132	135
Thiol-treated	–	141	145
	Fru-1,6-P_2 1 mM	165	165
	PEP 0.8 mM	129	125
	PEP 0.8 mM + Fru-1,6-P_2 1 mM	165	165

Fru-P_2ase, either as the untreated or as the thiol-treated enzyme, was added to the reaction media of assay procedures A and B as in section 2 except that DTT was omitted, and the Fru-1,6-P_2, $MgCl_2$ and PEP concentrations were raised by the additions indicated

Fig.1 shows the time course of Fru-P_2ase activity measured using the inactive (untreated) form of the enzyme in the presence of low, physiological concentrations of Fru-1,6-P_2 and Mg^{2+} and with DTT present as reductant to substitute for the light-driven reduction experienced by Fru-P_2ase in vivo. The increasing activity observed with time over the first few minutes (fig.1) represents the activation of Fru-P_2ase by DTT. The maximum rate was attained after only 15 min, yet is comparable with the rates measured under similar conditions (table 1) with a similar

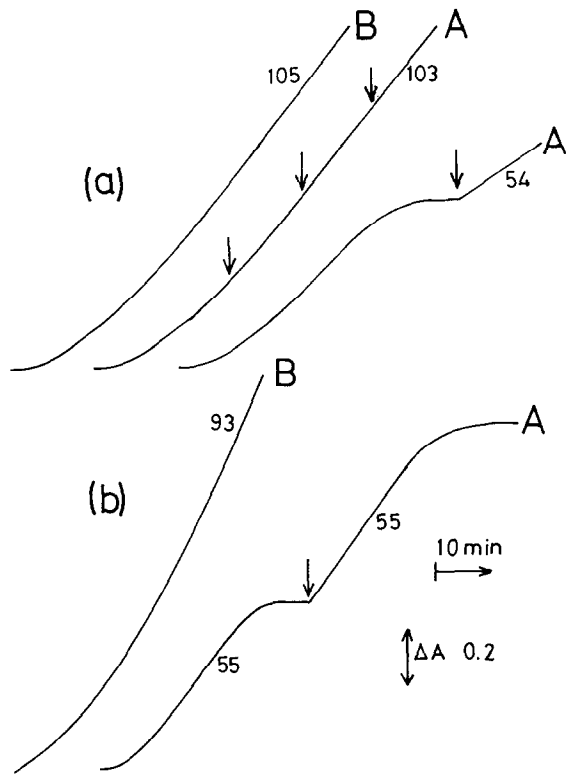

Fig.1. Time course of Fru-P_2ase activity showing the DTT-dependent activation. Either assay procedure A or B was used as indicated, with untreated purified Fru-P_2ase added to start the reactions: 0.04 μg (a); 0.08 μg (b). The arrows indicate the addition of Fru-1,6-P_2, 50 μM in (a) and 100 μM in (b). The rates of Fru-P_2ase activity are given alongside the traces, expressed as μmol Fru-6-P formed . min^{-1} . mg $protein^{-1}$. To facilitate comparison, the traces obtained with the two assay procedures A and B are presented so as to give a ΔA_{340} in the same direction, although A gave an increased and B a decreased ΔA_{340}.

Fru-P_2ase preparation pre-treated with DTT for 24 h [7]. Fig.1a shows that assay procedures A and B gave similar enzyme activities provided that, in assay procedure A, Fru-1,6-P_2 was replenished when it reached ~0.05 mM. When the Fru-1,6-P_2 was not replenished, enzyme activity declined until all the Fru-1,6-P_2 had been consumed. The unexpected consequence of this was that even if Fru-1,6-P_2 was subsequently returned to the reaction mixture full enzyme activity was not restored. It may be concluded from the results of fig.1a that it is possible to activate fully the Fru-P_2ase with DTT in a very short time so that it shows high activity at low concentrations of Fru-1,6-P_2 and Mg^{2+}, provided that the Fru-1,6-P_2 is always present during activation and subsequently does not fall below a mininal concentration. This is further illustrated in fig.1b where in assay procedure A an increased Fru-P_2ase concentration has consumed all of the Fru-1,6-P_2 before the Fru-P_2ase had become fully activated, and therefore before the maximum rate had been attained. A subsequent addition of Fru-1,6-P_2 did not allow the maximum rate to be attained (fig.1b). By contrast in the new assay procedure B the requirement for a continued presence of Fru-1,6-P_2 for the full activation of Fru-P_2ase is met automatically by the enzymatic regeneration of Fru-1,6-P_2 from F-6-P.

With both assay procedures A and B the higher the concentration of Fru-1,6-P_2 included in the reaction mixture the greater was the degree of activation and the shorter was the activation period (fig.2). When assay procedure B was used the Fru-1,6-P_2 concentration was kept constant during the assay period, but when assay procedure A was used the Fru-1,6-P_2 concentration declined continuously during the assay, and thus it was necessary to add Fru-1,6-P_2 periodically in order to maintain enzyme activity (fig.2). These additions were made whenever the Fru-1,6-P_2 reached 0.05 mM. When 0.2 mM Fru-1,6-P_2 was present initially, and assay procedure A was used, [Fru-1,6-P_2] fell from 0.2–0.05 mM, but the rate measured after activation was completed was always higher than the maximum rate measured when 0.1 mM Fru-1,6-P_2 was present throughout the assay period (assay procedure B) (fig.2). Thus the higher rates observed with the higher Fru-1,6-P_2 concentrations initially present are attributable to an effect of Fru-1,6-P_2 on the activation process itself rather than to a direct effect on the catalytic activity of the Fru-P_2ase.

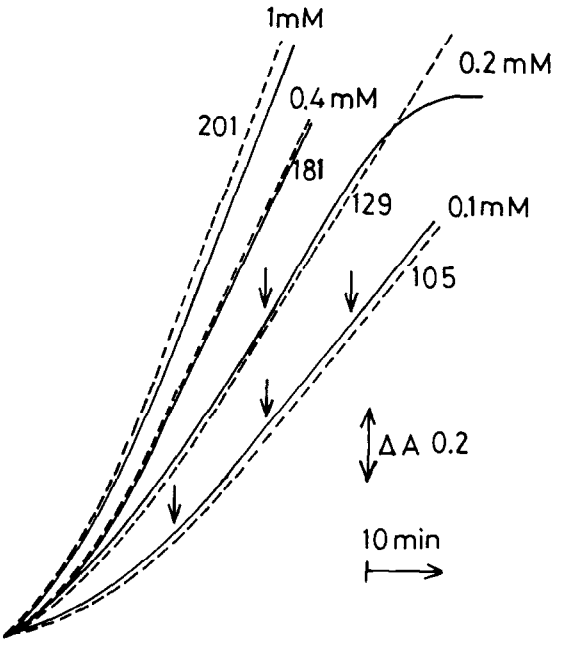

Fig.2. Effect of Fru-1,6-P_2 concentration on the activation of Fru-P_2ase. Either assay procedure A (——) or B (– – –) was used with the initial Fru-1,6-P_2 concentration indicated. The arrows indicate the addition of 50 μM Fru-1,6-P_2 (assay procedure A only). The rates of the Fru-P_2ase activity are given alongside the traces, expressed as μmol . min^{-1} . mg $protein^{-1}$. As in fig.1 the traces are presented to give a ΔA_{340} in the same direction.

4. Discussion

The main advantage of the new assay procedure for measuring Fru-P_2ase is that the Fru-1,6-P_2 is maintained at a constant steady-state level by continuous regeneration so that the activation process can be followed easily and that measurements at low Fru-1,6-P_2 concentrations are facilitated. Here, only the purified chloroplast Fru-P_2ase has been used, although the new assay procedure has obvious advantages in the study of the purified Fru-P_2ase from animal tissues and from plant cytosol, where Fru-1,6-P_2 is an inhibitor [12].

The main conclusion to be drawn from these results is that for the full DTT-dependent activation of the purified chloroplast Fru-P_2ase in a time similar to that required in the intact chloroplast [1] the only requirement during the activation period is for the presence of Mg^{2+} and Fru-1,6-P_2 at concentrations similar to those present in the chloroplast stroma [3,4].

On illumination the Fru-1,6-P_2 concentration in the chloroplast stroma increases from <0.1 mM to ~0.4 mM [3]. The present results (fig.2) show that in vitro this increase in [Fru-1,6-P_2] approximately halves the activation time and doubles the maximum rate. They also show that while [Fru-1,6-P_2] remains at ≳0.05 mM the maximum rate is maintained, but when Fru-1,6-P_2 is further depleted the Fru-P_2ase becomes partially deactivated. This sensitivity of purified Fru-P_2ase to the Fru-1,6-P_2 level in vitro indicates that Fru-1,6-P_2 has a modulating role on the activity of the enzyme in vivo, thus the Fru-P_2ase would become to some extent selfregulating.

The light-dependent activation of Fru-P_2ase in isolated intact chloroplasts is greatly inhibited by the inclusion of a high concentration of P_i in the suspending medium [9,13,14]. This effect, ascribed to a general loss of intermediates of the Calvin cycle from the stroma via the phosphate carrier in [14], can now be explained more specifically in terms of a depletion of stromal Fru-1,6-P_2 [15].

Here enzyme activation and enzyme activity have been allowed to occur simultaneously, whereas Fru-P_2ase had been incubated under a variety of conditions for activation, then a sample taken and added to the assay medium in [5–9]. The use of this preincubation period has helped to obscure the requirement for both Fru-1,6-P_2 and Mg^{2+} for a rapid activation since the Fru-1,6-P_2 in the preincubation medium soon became depleted, and consequently the enzyme became partially deactivated (see fig.1). The requirement for Ca^{2+} during preincubation noted in [8,9] can now be explained. When Mg^{2+} and Fru-1,6-P_2 were present in the preincubation mixture [9] Ca^{2+} would have inhibited the Fru-P_2ase activity [11] so as to prevent depletion of Fru-1,6-P_2 and the consequent deactivation of the enzyme. When Fru-1,6-P_2 was present in, but Mg^{2+} absent from, the preincubation mixture [8] Ca^{2+} would have substituted for Mg^{2+} as a divalent cation cofactor for activation without permitting an activity that would have depleted the Fru-1,6-P_2 present.

Acknowledgements

The author is grateful to Drs S. A. Charles and B. Halliwell for the purified enzyme, to Professor F. R. Whatley, Drs E. A. Newsholme and S. A. Charles for useful discussions, and to NATO for a Research Fellowship.

References

[1] Kelly, G. J., Zimmerman, G. and Latzko, E. (1976) Biochem. Biophys. Res. Commun. 70, 193–199.
[2] Wolosiuk, R. A. and Buchanan, B. B. (1977) Nature 266, 565–567.
[3] Lilley, R. McC., Chon, C. J., Mosbach, A. and Heldt, H. W. (1977) Biochim. Biophys. Acta 460, 259–272.
[4] Portis, A. R. jr, Chon, C. J., Mosbach, A. and Heldt, H. W. (1977) Biochim. Biophys. Acta 461, 313–325.
[5] Schürmann, P. and Wolosiuk, R. A. (1978) Biochim. Biophys. Acta 522, 130–138.
[6] Zimmerman, G., Kelly, G. J. and Latzko, E. (1976) Eur. J. Biochem. 70, 361–367.
[7] Charles, S. A. and Halliwell, B. (1980) Biochem. J. 185, 689–693.
[8] Hertig, C. and Wolosiuk, R. A. (1980) Biochem. Biophys. Res. Commun. 97, 325–533.
[9] Charles, S. A. and Halliwell, B. (1981) Cell Calcium in press.
[10] Miginiac-Maslow, M. and Hoarau, A. (1977) Plant Sci. Lett. 9, 7–15.
[11] Charles, S. A. and Halliwell, B. (1980) Biochem. J. 188, 775–779.
[12] Zimmerman, G., Kelly, G. J. and Latzko, E. (1978) J. Biol. Chem. 253, 5952–5956.
[13] Huber, S. C. (1979) Biochim. Biophys. Acta 545, 131–140.
[14] Heldt, H. W., Laing, W., Lorimer, G. H., Stitt, M. and Wirtz, W. (1981) Proc. Vth Int. Cong. Photosynthesis, Halkidiki, Sept. 1980 in press.
[15] Flügge, U. I., Freisl, M. and Heldt, H. W. (1980) Plant Physiol. 65, 574–577.